Multiplex CHO Mutagenesis System (A. W. Hsie)

Several mammalian cell mutation systems, especially those utilizing resistance to purine analogues such as 8-azaguanine and 6-thioguanine (TG) as a genetic marker, have been developed for quantitative mutagenesis and mutagen screening.

The selection for mutation induction to purine analogue resistance is based on the fact that the wild-type cells containing HGPRT activity are capable of converting the analogue to toxic metabolites, leading to cell death; the presumptive mutants, by virtue of the loss of HGPRT activity, are incapable of forming this detrimental metabolite and, hence, escape the lethal effect of the phosphorylated purine analogue.

The near-diploid CHO cell line has been chosen for our mutation assay, referred to as CHO/HGPRT system. We have used CHO cells because these are perhaps the best genetically characterized mammalian cells. They exhibit high cloning efficiency, achieving nearly 100% under normal growth conditions, and are capable of growing in a relatively well-defined medium on a glass or plastic substratum or in suspension, with a population doubling time of 12-14 hr. The CHO/HGPRT system has been well defined in terms of experimental parameters affecting quantitative mutagenesis and the system appears to fulfill the criteria for a specific locus mutation assay. It has been shown that this system can be used to determine the mutagenicity of various classes of organic chemicals, physical agents, volatile liquids and gases, metallic compounds, and the interactive effects between physical and chemical agents with high sensitivity.

It has long been recognized that CHO cells have a stable, easily recognizable karyotype of 20 or 21 chromosomes (depending on the subclone), and are suitable for studying chemical-induced chromosome and chromatid

aberrations and sister chromatid exchange in addition to cytotoxicity and gene mutation. Employing various carcinogenic/non-carcinogenic pairs we have found that it is feasible to expand the CHO/HGPRT system to include determination of chromosome aberration and SCE in addition to cytotoxicity and gene mutation, in a population of CHO cells treated with a given mutagen. This so-called Multiplex CHO Mutagenesis System permits simultaneous determination of four distinct biological effects.

Approach (methods). Protocols for determining cytotoxicity, gene mutation, chromosome aberration and sister chromatid exchange in CHO cells have been established in our laboratory.

Cell culture. For most studies, unless specified otherwise, we use a subclone of the CHO cell line, CHO-K_1-BH_4, which was isolated following selection in F12 medium containing aminopterin. Cells are routinely cultured in Ham's F12 medium containing 5% heat-inactivated (56°C, 30 min), extensively dialyzed fetal calf serum (medium F12FCM5) in glass bottles under standard conditions of 5% CO_2 in air at 37°C in an incubator humidified to 100%. Cells are removed with 0.05% trypsin for subculture and the number determined with a Coulter counter.

Treatment with chemical carcinogen-mutagen. We follow a standard procedure which is suitable for various agents. Briefly, CHO cells are plated at 5×10^5 cells/25 cm^2 plastic flask in medium F12FCM5. After a 16- to 24-hr growth period (cell number = $\sim 0.8\text{-}1.2 \times 10^6$ cells/flask), the cells are washed twice with saline G, and sufficient serum-free F12 medium is added to bring the final volume to 5 ml after the addition of various amounts of S-9 preparation (up to 1 ml) and 50 µl of mutagen, usually dissolved in dimethylsulfoxide. Mutagen and/or S-9 are omitted from some plates to provide controls. The S-9 fraction has been prepared from Aroclor

1254-induced male Sprague-Dawley rat livers in this laboratory according to standard protocol. After treatment with mutagen, cells are incubated for 5 hr, and washed three times with Saline G before 5 ml of F12FCM5 are added. Following incubation for an additional 19 hr, cells are trypsinized and plated for cytotoxicity, specific gene mutagenesis, chromosome aberration and sister chromatid exchange to be described below.

Cytotoxicity. The effect of a chemical mutagen on the cellular cloning efficiency is determined using the treated cells described above. For an expected cloning efficiency higher than 50%, 200 well-dispersed single cells are plated; for survival lower than this, the number of cells plated is adjusted to yield 100-200 surviving colonies after standard incubation in medium F12FCM5 for 7 days. At the end of the incubation period, the plates are fixed with 3.7% formalin, stained with a dilute crystal violet solution and the colonies enumerated. A cluster of more than 50 cells growing within a confined area is considered a colony. Control cultures, which do not receive mutagen, routinely give 80% or higher cloning efficiency under these conditions. The solvent and S-9 mix, either singly or in combination, do not affect the cloning efficiency. The effect of mutagen on the cloning efficiency is expressed as percent survival relative to the untreated controls. Weighted regression analysis is used to determine the characteristics of the dose-response line for both cytotoxicity and mutagenicity.

Specific gene mutagenesis. For the determination of specific locus mutation, the treated cells are allowed to express the mutant phenotype in F12 medium for 7-9 days, at which time the expression of mutation induction reaches a maximum and is maintained thereafter. Routine subculture is performed at 2-day intervals during the expression period before the cells are plated for selection in hypoxanthine-free F12FCM5 containing 10 μM of TG.

The cells are plated at a density of 2.0×10^5 cells/100 mm plastic dish in each of 5 plates for a total of 10^6 cells. These conditions permit ~100% mutant recovery in reconstruction experiments. After 7 to 8 days in the selective medium, the drug-resistant colonies develop; they are then fixed, stained, and counted. Mutation frequency is calculated based on the number of drug-resistant colonies per 10^6 clonable cells at the end of the expression period.

Chromosome aberration.* For this study we follow the procedure for detecting chromosome and chromatid aberrations in cell culture as adapted for use in CHO cells by us. Briefly, 16 hours after rescuing the treated cells, Colcemid (2×10^{-7} M final concentration) is added to the culture for 2 hr. The loosely attached cells are removed with the growth medium; after trypsinization (at 37°C for 5 min) of the monolayer, all the cells are combined and centrifuged in an International Clinical Centrifuge (1000 rpm for 5 min). The cell pellet is resuspended in 10 ml of 75 mM KCl for 5 min in a 37°C waterbath, and then centrifuged for 5 min. After fixing in methanol:glacial acetic acid (3:1), the cells are kept at 4°C overnight. The cells are then washed three times with fresh fixative and the cell pellet suspended in a small amount of fixative (0.4-0.5 ml). Two to three drops of this cell suspension are dropped onto chilled, wet slides. After drying in the air, the slides are stored at 4°C for 48-72 hr, and then stained with 2% Giemsa (in 0.01 M sodium phosphate buffer, pH 6.8) for 5 min. Chromosome and chromatid aberrations in the form of chromatid gaps, chromatid or chromosome breaks, translocation, ring formation, dicentrics, fragmentation are scored using a Zeiss microscope.

Sister chromatid exchange.* The technique for measuring sister

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*Not to be used in this study.

chromatid exchange, as adapted in this laboratory, is used. Briefly, after removal of mutagen from the cells, 10 ml of medium F12FCM5 containing 10 μM of 5-bromodeoxyuridine (BUdR) is added to the treated culture. The procedures are done under gold light and the cultures are subsequently incubated in complete darkness. After incubation for 24-30 hr, which permits two rounds of DNA replication in the presence of BUdR, Colecmid (2×10^{-7} M final concentration) is added to the culture for 2 hr. The cells are collected and the slides prepared as described above for chromosome aberration studies. After storage in the cold (4°C) for 48-72 hr, the slides are stained in Hoechst 33258 and dried in air. Two to three drops of McIlvaine's buffer (pH 8.0) are placed on the slides and they are exposed to irradiation from two 15 watt fluorescent black lights (General Electric) 5 cm from the surface of slides for 15 min at 50°C. The slides are rinsed in distilled water and stained with 2% Giemsa (in sodium phosphate buffer 0.01 M, pH 6.8) for 15 min before washing thoroughly in tap water and air drying. Sister chromatid exchanges are scored using a Zeiss microscope.

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